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Abstract

Starter cultures have an essential role in cheese production contributing to desired acid production and formation of aroma- and flavour compounds during cheese maturation. Residues of antimicrobial drugs might reduce the activity of dairy starter cultures and subsequently cause impaired cheese quality. The incidence of prescription of antimicrobial drugs to dairy cows in Sweden is low. In addition, the maximum residue levels (MRL) of antimicrobial drugs in bovine milk are regulated. However, studies suggest that residues of antimicrobial drugs might impact on starter culture bacteria even at concentrations below the MRL. The aim of this study was to investigate how penicillin G, oxytetracycline and trimethoprim-sulfadiazine affect the bacteria in commercial mesophilic starter cultures at concentrations corresponding to their respective MRL values. The effect of the individual antimicrobial drugs on the activity of the starter cultures was investigated by measuring pH during fermentation. In addition, changes in starter culture composition was analysed by selective cultivation on x-gal calcium citrate agar. Oxytetracycline at 100 ppb had a significant effect on the fermentation in 2 out of 3 starter cultures, while no significant effect on the culture activity was observed at 4 ppb penicillin G or 100 ppb trimethoprim-sulfadiazine. Penicillin G at 4 ppb had a significant effect on numbers *Lactococcus lactis* spp. *lactis/cremoris* in 1 out of 3 starter cultures while no significant effect was observed for *Lactococcus lactis* spp. *lactis* biovar. diacetylactis or *Leuconostoc* spp. Oxytetracycline at 100 ppb had a significant effect on *Lactococcus lactis* spp. *lactis* biovar. diacetylactis in 2 out of 3 starter cultures, whereas *Leuconostoc* spp. and *Lactococcus lactis* spp. *lactis/cremoris* were significantly inhibited in 1 out of 3 starter cultures. 100 ppb trimethoprim-sulfadiazine had no clear effect on starter culture bacteria. Further studies are necessary to confirm the results of the study.

Keywords: mesophilic starter cultures, lactic acid bacteria, aroma producing bacteria, acid producing bacteria, penicillin G, oxytetracycline, trimethoprim, sulfadiazine

Sammanfattning

Starterkulturer fyller en viktig funktion i osttillverkning genom att bidra till syraproduktion och bildandet av arom- och smakämnen under ostmognaden. Rester av antimikrobiella läkemedel i mjölk kan orsaka minskad aktivitet hos starterkulturbakterier med försämrad produktkvalitet som följd. Förskrivningen av antimikrobiella substanser till mjölkkor i Sverige är låg. Dessutom regleras förekomsten av antimikrobiella läkemedelsrester i mjölk av det högsta tillåtna gränsvärdet (s.k. maximum residue limit, MRL) för olika substanser. Studier visar emellertid att rester av antimikrobiella läkemedel kan påverka starterkulturbakterier vid lägre koncentrationer än de aktuella gränsvärdena. Syftet med den här studien var att undersöka hur penicillin G, oxytetracyklin och trimetoprim-sulfadiazin påverkar fermenteringshastigheten för mesofila starterkulturer för osttillverkning vid koncentrationer motsvarande gränsvärdet för respektive substans. I studien analyserades restsubstansernas effekt på aktiviteten hos starterkulturerna genom att mäta pH under fermenteringsprocessen. Därutöver analyserades förändringar i starterkulturernas sammansättning genom selektiv odling på x-gal-kalciumcitratagar. Oxytetracyklin (100 ppb) hade en signifikant effekt på fermenteringen i 2 av 3 starterkulturer, medan 4 ppb penicillin G och 100 ppb trimethoprim-sulfadiazin inte hade någon signifikant effekt. Penicillin (4 ppb) hade en signifikant effekt på antalet *Lactococcus lactis* spp. *lactis/cremoris* i 1 av 3 starterkulturer, medan en effekt på antalet *Lactococcus lactis* spp. *lactis* biovar. diacetylactis eller *Leuconostoc* spp. saknades. Oxytetracyklin (100 ppb) orsakade signifikant inhibering av *Lactococcus lactis* spp. *lactis* biovar. diacetylactis i 2 av 3 starterkulturer liksom för *Leuconostoc* spp. och *Lactococcus lactis* spp. *lactis/cremoris* i 1 av 3 starterkulturer. Trimethoprim-sulfadiazine (100 ppb) hade ingen effekt på de studerade mjölksyrabakterierna. Fortsatta studier rekommenderas för att säkerställa resultatet i studien.

Nyckelord: mesofila starterkulturer, mjölksyrabakterier, arombildande bakterier, syrabildande bakterier, penicillin G, oxytetracyklin, trimetoprim, sulfadiazin

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Abbreviations

AD	Antimicrobial drug
ANOVA	One-way analysis of variance
β-gal	β-galactosidase
CFU	Colony forming unit
EMA	European medicines agency
MIC	Minimum inhibitory concentration
MRL	Maximum residue level
PPB	Parts per billion
PBP	Penicillin G binding protein
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

1. Introduction

1.1 Aim

The aim of the study was to investigate how commercial mesophilic starter cultures for cheese production are affected by residues of antimicrobial drugs in milk. The hypothesis was that antimicrobial drugs would have different effects depending on concentration and mechanism of action.

1.2 Delimitations

The study was performed as an explorative pilot study. The investigated concentrations of antimicrobial drugs were limited to the European medicines agency´s elaborated maximum residue levels. The study included the three most commonly used antimicrobial drugs in dairy production in Sweden, and three commercial starter cultures that are of interest in the production of long ripened cheese. The effect of each antimicrobial drug on fermentation and composition of the different starter cultures was investigated once per starter culture.

2. Background

2.1 The use of antimicrobial drugs in dairy cows

2.1.1 Situation in Sweden

The prescription of antimicrobial drugs to dairy cows in Sweden is low. The incidence of prescriptions decreased from 2001 to 2016 but has varied to a larger extent the recent years. The reduction in the number of prescriptions can partly be explained by improved animal health and improved treatment strategies. However, impaired reporting of cases of animal treatments could also contribute to the reduced prescription incidence (Växa 2020).

A majority of all prescriptions of antimicrobial drugs to dairy cows in Sweden can be derived to mastitis, i.e., infection and inflammation of the mammary gland. In 2020, 58 % of all prescriptions of antimicrobial drugs was for treatment of this disease. However, the percentage of prescriptions derived to mastitis has decreased lately, and for comparison, in 2012, 70 % of all prescriptions of antimicrobial drugs to dairy cows in Sweden was for treatment of mastitis. The primary choice for antimicrobial treatment of mastitis is a β-lactam, e.g., penicillin G. In Sweden, β-lactams were prescribed in 92 % of all mastitis cases reported in 2020, followed by 6 % with a sulphonamide and trimethoprim in combination. In Västerbotten, β-lactams were prescribed in 96.8 % of all mastitis cases (Växa 2020).

Simultaneously, the percentage of antimicrobial prescriptions related to other diseases has increased from 7 % to 15 % of total. Other diseases where antimicrobial drugs are prescribed include leg and claw, lung, skin and gastric infections. Although β-lactams are still the most common antimicrobial drugs to be prescribed in dairy cows, the percentage of tetracycline prescriptions is increasing in non-mastitis cases. The increased use of antimicrobial drugs in treatment of other diseases than mastitis has subsequently led to an increase in the prescription of tetracyclines. In 2020, tetracyclines were prescribed in 12-20 % of all treatments related to other diagnoses than mastitis in Sweden (Växa 2020).

2.1.2 Antimicrobial drugs

Antimicrobial drugs are compounds that inhibit bacterial growth, either by a bacteriostatic effect, which prevents the bacterial growth, or by a bactericidal effect which kills the bacteria (Klein & Cunha 1995). Antimicrobial drugs of natural origin are commonly referred to as antibiotics. Antimicrobial drugs that are produced by chemical synthesis is referred to as chemotherapeutic drugs. Antibiotics were initially considered to consist of low molecular weight molecules or metabolites produced by microorganisms in response to competition for nutrients in the habitat. However, recently a more complex function of antibiotics in metabolic and signalling systems of microorganisms has been suggested (Nelson & Levy 2011).

β-lactams

All β-lactam antimicrobial drugs possess a β-lactam ring consisting of a cyclic amide. Antimicrobial drugs within the group of β-lactams can be divided into cephalosporins, penicillins, monobactams and carbapenems (Sykes & Papich 2014). β-lactams are of high importance in veterinary and human medicine and they represent a substantial part of the total production of antimicrobial drugs globally (Madigan 2003). Penicillins include the natural penicillin G but also semi-synthetic antimicrobial drugs such as amoxicillin and methicillin (Madigan 2003). Penicillin G was the first β-lactam antibiotic to be discovered, and it was detected by Alexander Fleming in 1928. It is produced by the fungus *Penicillium chrysogenum* (Madigan 2003), a narrow spectrum antibiotic that primary inhibits gram-positive bacteria. However, gram-negative anaerobes can also be sensitive to penicillin G (Castle 2007). Penicillin G is a common choice for treatment of mastitis in dairy cows (Madigan 2003).

Penicillin G inhibits cell wall synthesis in bacteria by binding to enzymes called transpeptidases that catalyse transpeptidation, an important part of cell wall synthesis in bacteria. The transpeptidases, also called penicillin G binding proteins (PBPs), bind strongly to penicillin G resulting in alterations in the cell wall synthesis. This results in a weaker cell wall without cross-linkages. In addition, the complexes of penicillin G and PBPs increase the level of cell wall-digesting autolysins, leading to cell wall degradation. Normally, differences in osmotic pressure on the in- and outside of the cell eventually cause cell lysis. The action of penicillin G is substantially reduced in organisms where the enzyme β-lactamase is present (Madigan 2003).

Tetracyclines

Tetracyclines are broad-spectrum bacteriostatic antimicrobial drugs that inhibit aerobic, anaerobic, gram-positive and gram-negative bacteria (Klein & Cunha 1995). There are both natural and synthetic tetracyclines. The common structure among tetracycline compounds is the naphthacene ring system. Alterations within the naphthacene ring system distinguish different tetracycline analogues. Oxytetracycline receives an inhibitory effect by binding to the 30S ribosomal subunit in bacteria, which results in inhibition of protein synthesis. Oxytetracycline normally has a bacteriostatic, rather than bactericidal, effect (Klein & Cunha 2011).

The first tetracycline compound to be discovered was Aureomycin, a natural antimicrobial compound produced by *Streptomyces aureofaciens*. The second tetracycline to be discovered was Terramycin, produced by *Streptomyces rimosomus*. Terramycin possesses a higher water solubility and bioactivity than Aureomycin and was considered to be a more efficient inhibitor of bacteria. Subsequently, the molecular structures of Aureomycin and Terramycin were established. Terramycin had an additional hydroxyl group and was later named oxytetracycline. Aureomycin had an additional chlorine atom and was given the name chlortetracycline (Nelson & Levy 2011). Tetracyclines can also be produced synthetically, for example doxycycline and minocycline. In comparison to natural tetracyclines, synthetically produced tetracyclines are more stable, and efficiently absorbed orally and secreted via the hepatic system (Klein & Cunha 1995).

Trimethoprim and sulphonamides

Trimethoprim and sulphonamides are synthetically produced antimicrobial drugs that inhibit folic acid metabolism in bacteria. Sulphonamides, due to their structural similarity to para-aminobenzoic acid (PABA), act as competitive inhibitors in the folic acid metabolism cycle. A reduction in folic acid impairs the synthesis of purines, compounds which are essential for DNA synthesis (Katla et al 2001). Since animals receive folic acid through diet, only the synthesis of purines in bacterial cells is affected by treatment with trimethoprim and sulphonamides. A combination of trimethoprim and sulfadiazine has a synergistic negative effect on the folic acid metabolism in bacteria. Individually, trimethoprim and sulphonamides are bacteriostatic however, the combination is bactericidal (Sykes and Papich 2014). Resistance to trimethoprim and sulfadiazine occur in bacteria that receive folic acid from exogenous sources (Madigan 2003).

2.1.3 Antimicrobial drug residues in milk

Antimicrobial drugs that are used in the treatment of dairy cattle can be transferred to the milk resulting in antimicrobial drug residues in dairy food products (Chiesa et al 2020). The maximum residue levels (MRLs) for penicillin G, oxytetracycline,

trimethoprim and sulfadiazine in bovine milk, respectively, are presented in table 1. The MRLs have been elaborated by the Committee for veterinary medicinal products at the European medicines agency (EMA). The limits have been developed with regard to the potential health and safety risks that are connected to the drugs but may also consider the effect on starter bacteria in dairy products (European Medicines Agency, 2008). Common commercial tests for detection of antimicrobial drug residues in milk include the microbial inhibitor test Delvotest® (DSM food specialties, Delft) and Betastar® (Neogen, Lansing), which is based on the binding ß-lactams to a receptor protein. Delvotest® provides detection of a broad spectrum of antimicrobial drugs that can be found in milk, including penicillin G, sulfadiazine, oxytetracycline and trimethoprim. Delvotest® is used by for example farmers, dairies and laboratories. According to the manufacturer, the detection limit of Delvotest® for penicillin G and sulfadiazine is below or corresponding to their respective MRL while the detection limit for oxytetracycline is above MRL. No information is presented regarding the detection limit for trimethoprim (DSM food specialities 2011). BetaStar® S will only provide detection of β-lactams in milk and is utilized by for example farmers, dairies and laboratories. According to the manufacturer, BetaStar® S has a detection limit below or corresponding to the MRL for different β-lactams (Neogen 2022).

Table 1. Maximum residue levels in bovine milk for antimicrobial drugs included in the study

Antimicrobial drug	Maximum residue level (MRL) in bovine milk (ppb)
Penicillin G	4 [1,2]
Oxytetracycline	100 [2,3]
Trimethoprim	50 [4]
Sulfadiazine	100 [5]

[1] European Medicines Agency 2008; [2] World Health Organisation 2018; [3]European Medicines Agency 1996; [4] European Medicines Agency 2002; [5] European Medicines Agency 1995

2.1.4 Effect of antimicrobial drug residues on cheese quality

Residues of antimicrobial drugs in milk are undesirable in cheese manufacturing due to the potential reduction in starter culture activity resulting in impaired acid formation (Chiesa et al 2020; Cogan 1972). Impaired acid formation decreases the enzymatic activity of the rennet which subsequently causes slower milk curdling (Chiesa et al 2020). Reduced starter culture activity may also impair cheese maturation (Chiesa et al 2020). The resulting cheese will possess a low acidity and might be perceived as pasty (Cogan 1972). Residues of antimicrobial drugs are

likely to have an impact on starter culture activity even at concentrations below MRL (Chiesa et al 2020; Katla et al 2001). Occurrence of antimicrobial residues in milk can in that sense cause impaired cheese quality and economical losses even at low concentrations, which may go undetected in the control of the dairy silo milk (Chiesa et al 2020). A concentration of 6 ppb penicillin in milk can inhibit starter culture bacteria substantially resulting in slower acid development (European medicines agency 2008).

2.2 Starter cultures and their role in dairy production

2.2.1 Starter cultures in cheese manufacture

Starter cultures are widely used in the production of dairy products. A common field of application is cheese manufacture, where starter cultures are used in the production of different cheeses, e.g., Cheddar, Gouda and Feta cheese. Starter cultures for cheese production can be classified as mesophilic and thermophilic starter cultures depending on the optimal growth temperature of the lactic acid bacteria (Walstra et al 2006). Mesophilic starter cultures mainly consist of one or several strains of lactic acid bacteria. Bacteria in mesophilic starter cultures posess optimal growth at 25-30˚C. This can be compared to bacteria in thermophilic starter cultures that posess optimal growth at 40-45˚C (Engels & Dusterhoft 2017). Common bacteria in thermophilic starter cultures are *Streptococcus thermophilus* in combination with either *Lactobacillus helveticus* or *Lactocbacillus delbrueckii* spp. *lactis*. Common bacteria in mesophilic starter cultures, which were in focus in this study, are *Lactococcus* spp. and *Leuconostoc* spp (Walstra et al 2006).

Addition of starter cultures to the milk during cheese production makes it possible to control the bacterial fermentation of lactose to lactic acid. The rate of the lactose fermentation is of importance for cheese curd formation and a rapid acid production is necessary to prevent undesired cheese deterioration by spoilage and/or pathogenic bacteria. Starter cultures also have substantial influence on cheese maturation. During cheese maturation, starter bacteria tend to become less viable and undergo autolysis, resulting in the release of enzymes to the cheese curd. Some of these enzymes catalyse proteolytic and lipolytic processes in the cheese resulting in formation of aroma- and flavour contributing compounds (Engels & Dusterhoft 2017).

2.2.2 Lactic acid bacteria

Lactic acid bacteria (LAB) can be divided in hetero- and homofermentative LAB. Homofermentative LAB produce lactic acid as the only fermentation product while heterofermentative LAB also produce other products such as citrate, ethanol, carbon dioxide and lactate (Madigan et al. 2003). LAB generally rely on carbohydrate metabolism for energy supply and it is therefore necessary that carbohydrates are present in the growth environment. In addition, LAB have a high nutritional demand due to an insufficient biosynthetic capacity. Important components of growth media for LAB include amino acids, purines, pyrimidines and vitamins (Madigan et al. 2003).

Main properties of *Lactococcus* spp. in dairy fermentations is lactic acid production and flavour production as a result of metabolization of milk proteins. (Mills et al. 2011). *Leuconostoc* spp. are able to ferment citrate into diacetyl which contributes to a butter-like flavour in cheese and butter products. *Leuconostoc* spp. also produce carbon dioxide which is necessary for cheese eye formation (Holland and Liu 2011). Characteristics of *Leuconostoc* spp. and *Lactococcus* spp. are presented in table 2.

Table 2. Characteristics of LAB commonly present in mesophilic starter cultures

Mesophilic bacteria	*Lactococcus lactis* spp. *cremoris*	*Lactococcus lactis* spp. *lactis*	*Lactococcus lactis* spp. *lactis biovar.* diacetylactis	*Leuconostoc* spp.
Cell shape[1]	Cocci	Cocci	Cocci	Cocci
Sugar fermentation[1]	Homoferm.	Homoferm.	Homoferm.	Heteroferm.
Optimal growth[2]	25-30˚C	25-30˚C	25-30˚C	25-30˚C
Growth at 10[1]	+	+	+	+
Growth at 45[1]	-	-	-	-
Citrate metabolism[1]	-	-	+	+
Diacetyl production[1]	-	-	+	+
CO_2 production[3, 4]	-	-	+	+
Aerotolerance[3, 4]	+	+	+	+
Gram positive[1]	+	+	+	+

[1] Walstra et al 2006; [2] Engels and Dusterhoft 2017; [3] Holland and Liu 2011; [4] Mills et al 2011

2.2.3 Single and multiple-strain cultures

Starter cultures can be classified as single- and multiple-strain cultures. Single-strain mesophilic starter cultures contain only one strain of a bacterium, typically *Lactococcus lactis* spp. *cremoris*. Multiple-strain cultures contain more than one strain and can be either defined or undefined. which refers to whether the bacterial strain composition is established or not. An undefined starter culture has a dynamic species composition that evolves naturally after time (Walstra et al 2006).

Single-strain mesophilic starter cultures contain one strain of a bacterium, typically *Lactococcus lactis* spp. *cremoris* but there are also cultures which consist of *Lactococcus lactis* spp. *lactis* biovar. diacetylactis. Multiple-strain mesophilic starter cultures contain more than one strain of *Lactococcus lactis* spp. *cremoris* and/or *Lactococcus lactis* spp. *lactis*. These strains are often combined with *Leuconostoc mesenteroides* spp. or *Lactococcus lactis* spp. *lactis* biovar. diacetylactis (Walstra et al 2006).

Multiple-strain mesophilic starter cultures can be named as either O, L, DL or D-cultures depending on what species that are present in addition to *Lactococcus lactis* spp. *lactis* and *Lactococcus lactis* spp. *cremoris* (table 3). L-cultures contain *Leuconosto*c spp., D-cultures contain *Lactococcus lactis* spp. *lactis* biovar. diacetylactis and DL-cultures contain both *Leuconostoc* spp. and *Lactococcus lactis* spp. *lactis* biovar. diacetylactis. O-cultures only contain *Lactococcus lactis* spp. *lactis* and/or *cremoris* (Walstra et al 2006).

Table 3. Lactic acid bacteria in L, DL, D and O-cultures

	Aromatic			Non-aromatic
Lactic acid bacteria	L	DL	D	O
Lactococcus lactis spp. *lactis*[1]	+	+	+	+
Lactococcus lactis spp. *cremoris*[1]	+	+	+	+
Lactococcus lactis spp. *lactis* biovar. diacetylactis[1]		+	+	
Leuconostoc mesenteroides spp.[1]	+	+		

[1] Walstra et al 2006

2.2.4 Determination of starter culture composition

Cultivation on x-gal calcium citrate agar makes it possible to differentiate between aroma- and acid producing bacteria in mesophilic starter cultures. This since aroma producing bacteria are able to ferment citrate resulting in clear zones in the agar. Acid producing bacteria lack citrate fermentation and their colonies will thus appear without the clear zones in the agar. By this, aroma producing *Leuconostoc* spp. and *Lactococcus lactis ssp. lactis* biovar. diacetylactis can be distinguished from acid producing *Lactococcus lactis* ssp. *lactis* and *Lactococcus lactis* ssp. *cremoris* in mesophilic starter cultures (Friedrich and Lenke 2006).

Leuconostoc spp. and *Lactococcus lactis* ssp. *lactis* biovar. diacetylactis can further be differentiated due to differences in β-galactosidase (β-gal) activity (Mathot et al 1993; Hemme & Foucaud-Scheunemann 2003). β-gal is an enzyme encoded by the *lacZ* gene in for example *Leuconostoc* spp. and *Lactococcus* spp. These species are therefore able to metabolise lactose to glucose and galactose (Juers et al 2014). The substance 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (x-gal) is a lactose analogue which can be hydrolysed by β-gal resulting in 5-bromo-4-chloro-indoxyl (Mathot et al 1994). The x-gal subsequently dimerizes resulting in an insoluble, blue-coloured product. β-gal-positive *Leuconostoc* spp. will thus appear as blue colonies due to the capacity to hydrolyse x-gal and β-gal negative *Lactococcus* spp. will appear as white colonies (Mathot et al 1994; Hemme & Foucaud-Scheunemann 2003).

3. Material and methods

3.1 Method development

To investigate how the mesophilic starter cultures are affected by antimicrobial drug residues in the milk, the study was divided into two experiments. A starter culture activity test was performed, where the fermentation of the starter culture in milk with residues of antimicrobial drugs was compared to the fermentation of the same starter culture in milk without residues of antimicrobial drugs. In addition, a starter culture composition test was performed, where the composition of acid- and aroma producing bacteria in the fermented milk samples was evaluated. This part was to investigate if the presence of the antimicrobial drugs affected acid- and aroma producing bacteria to various extent. The methods used in the study are further developments of Norrmejerier´s methods for culture activity test and cultivation of aroma- and acid producing bacteria, respectively. Initially, a substantial part of the work was devoted to set up and optimise the methods for use in this project. Aspects that were considered during the elaboration of the experimental procedure for the fermentation- and composition tests are presented in table 4.

Table 4. Different aspects that were considered during each step of the development of the starter culture activity and composition tests

Test	Step	Important aspects to consider
Starter culture activity	Reconstitution and heat treatment of skim milk powder	Reconstitution of skim milk powder, heat treatment type, length and temperature
	Preparation of antimicrobial drug solutions	Water solubility, stock solution preparation, shelf life/inactivation
	Preparation of milk samples	Dilution of starter culture, total sample volume, antimicrobial drug volume and concentration, equipment for measurement of pH, sterile handling
	Incubation/ pH measuring	Starting time, total incubation time, water bath temperature, pH measurement (frequency, manually/automatic, sterile handling)
Starter culture composition	Preparation of media	Melting temperature, avoiding air bubbles, transportation and handling of ready-made citrate agar plates from the Department of laboratory medicine at Norrland's University hospital, Umeå
	Preparation of dilution series	Contamination, pipetting accuracy
	Isolation of bacteria	Selection of fermented milk dilutions, volume media/plate, media temperature, application technique, sterile handling
	Incubation	Length, temperature, oxygen conditions
	Colony examination	Detection of contamination, examination technique (microscope)

3.2 Starter culture activity test

3.2.1 Starter cultures

The activity of three commercial mesophilic multiple-strain-DL-starter-cultures were investigated in the study. All starter cultures were obtained from Christian Hansen Holding A/S (Hoersholm, Denmark) The composition of the three starter cultures was similar (table 5). However, strain composition and the ratio between the species in the different cultures were unknown. Starter culture A and C were freeze-dried and added directly to the reconstituted milk whereas starter culture B was frozen at -18˚C and thawed at 20.5˚C in a water bath 30 minutes prior to use.

Table 5. Bacterial composition of the three investigated mesophilic starter cultures according to the manufacturer

Genus	**Species**	
	Aromatic	Non-aromatic
Lactococcus spp.	*Lactococcus lactis* spp. *lactis* biovar. diacetylactis	
		Lactococcus Lactis spp. *lactis*
		Lactococcus Lactis spp. *cremoris*
Leuconostoc spp.	Not specified by manufacturer	

3.2.2 Antimicrobial drugs

Antimicrobial drugs included in the fermentation test were penicillin G, oxytetracycline, trimethoprim and sulfadiazine. The two latter were added in combination to mimic the combinatory drug trimethoprim-sulfadiazine, at a ratio of 1:5. All substances were provided from the manufacturer Sigma Aldrich (Saint Louis, Missouri, USA). Penicillin G and oxytetracycline were dissolved in water whereas trimethoprim and sulfadiazine were dissolved in 70 % ethanol. Investigated concentrations corresponded to the respective MRLs in bovine milk, although the concentration of the combinatory drug consisting of trimethoprim and sulfadiazine was based on the MRL for sulfadiazine. Antimicrobial group and chemical name of penicillin G, oxytetracycline, trimethoprim and sulfadiazine are presented in table 6.

Table 6. Antimicrobial drug group and chemical name of penicillin G, oxytetracycline hydrochloride, trimethoprim and sulfadiazine

Common name	Antimicrobial drug group	Chemical name
Penicillin G	β-lactams	Benzylpenicillin sodium salt
Oxytetracycline hydrochloride	Tetracyclines	5-Hydroxytetracycline hydrochloride
Trimethoprim	Trimethoprim	2,4-Diamino-5-(3,4,5-trimethoxybenzyl) pyrimidine
Sulfadiazine	Sulphonamides	N^1-(Pyrimidin-2-yl)sulfanilamide, 4-Amino-N-(2-pyrimidinyl)benzenesulfonamide

3.2.3 Preparation

Skim milk

Skim milk powder, 84.5 g, was reconstituted in 850.0 g distilled water in an autoclaved 1000 ml bottle followed by proper mixing. The reconstituted skim milk was heat-treated at 93˚C in a water bath for 30 minutes and thereafter kept at 4˚C.

Penicillin G and oxytetracycline stock solutions

1 mg/ml stock solutions were prepared by weighing 50.0 mg antimicrobial drug in 50.0 mg autoclaved distilled water. The stock solution was divided in 1 ml aliquots and kept at -18˚C until use.

Trimethoprim-sulfadiazine 1:5 stock solution

The preparation of a trimethoprim-sulfadiazine 1:5 stock solution aimed to receive a final concentration of 1 mg/ml of sulfadiazine. Initially, 1.2 mg/ml stock solutions were prepared for trimethoprim and sulfadiazine, individually, and 60.0 mg trimethoprim and sulfadiazine, respectively, were resolved in 50.0 ml ethanol 70 %. Trimethoprim stock solution (4 ml of 1.2 mg/ml) and sulfadiazine stock solution (20 ml of 1.2 mg/ml) were pipetted to a 50 ml Falcon tube followed by gentle mixing. The trimethoprim-sulfadiazine 1:5 stock solution was further divided in 1 ml aliquots, and the aliquots were kept at -18˚C until use.

3.2.4 Inoculation of milk

Reconstituted skim milk was temperated at 20.5˚C for 2 h before initiation of the experiment. If starter culture B was used, it was initially thawed in a water bath at 20.5˚C for 30 minutes. An aliquot of the antimicrobial drug stock solution was thawed at room temperature for 30 minutes and then diluted according to figure 1.

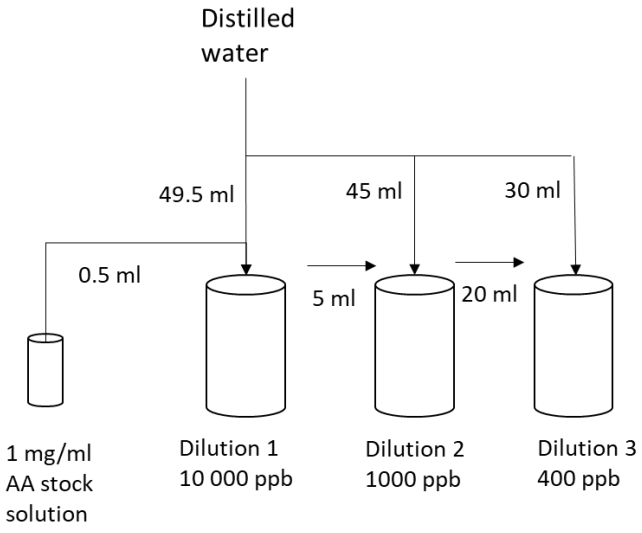

Figure 1. Illustration of the dilution series of antimicrobial drugs, in this example penicillin G, performed in connection to the starter culture activity test.

Four milk samples (1, 2, 3 and 4) were prepared by pipetting 270 ml reconstituted skim milk to four e-pistons. 500 ml reconstituted skim milk was transferred to a sterile 1000 ml glass bottle (solution A). 199 ml reconstituted skim milk was pipetted to a sterile 200 ml glass beaker (solution B). The starter culture was opened with a sterile scissor and poured into solution B followed by gentle mixing in approximately 10 minutes until the starter culture became completely dissolved in the reconstituted skim milk. 1 ml of solution A was then pipetted to solution B followed by proper mixing. 30 ml of solution B was pipetted to the sample 1, 2, 3 and 4, and 3 ml of sterile distilled water was added to sample 1 (negative control). Antimicrobial drug solution was added to sample 2, 3 and 4 according to table 7.

Table 7. Volume and concentration of the antimicrobial drug dilutions added to milk samples to receive the final concentration of the different antimicrobial drugs in the milk

Antimicrobial drug	Concentration (ppb)	Volume (ml) of the antimicrobial drug dilution in 300 ml milk	Concentration (ppb) of the antimicrobial drug in milk
Penicillin G	400	3	4
Oxytetracycline	10 000	3	100
Trimethoprim-sulfadiazine	10 000	3	100

3.2.5 Incubation

Samples were incubated in a water bath at 20.5˚C. The pH-meter (Mettler Toledo InLab® Max Pro-ISM) was calibrated prior to the first measurement. All sets of measurements started with the negative control to avoid contamination. The electrode was cleaned properly with distilled water and ethanol 70 % between the measurements.

The second pH measurement was performed after approximately 750 minutes (12.5 hours) and then repeatedly at two occasions per hour. The incubation lasted for approximately 16-20 hours until the negative control had reached pH 4.55-4.60. All measurements were performed manually.

After finishing the incubation, measurement data (pH) of the negative control and the replicates were plotted to receive graphs illustrating starter culture activity during fermentation. A statistical analysis were performed to investigate if there were significant differences in pH between the negative control and the replicates during fermentation. In the statistical analysis, the difference in fermentation was analysed by comparing the mean pH of the negative control with the mean pH of the replicates. All measurement data except at time 0 were included in the analysis.

3.3 Starter culture composition

3.3.1 Cultivation media

The cultivation of mesophilic bacteria in fermented milk samples was performed on x-gal-calcium citrate agar with added bacteria filtrate and citrate solution. The calcium citrate agar was delivered from the Department for laboratory medicine at Norrland's university hospital in Umeå, Sweden. Bacteria filtrate and citrate solutions were delivered in glass tubes from SSI Diagnostica (Hilleröd, Denmark), and stored at 4˚C until use. A 4 mg/ml solution of x-gal (VWR, Radnor, Pennsylvania, USA) and DMSO/NMP was obtained and stored in dark conditions at room temperature.

3.3.2 Preparation

Peptone water was prepared by mixing 1 g peptone (Oxoid Ltd, Basingstoke, Hampshire, England) and 1000 ml distilled water. The peptone water was distributed in 150 ml aliquots, autoclaved and stored at 4˚C until use.

The calcium citrate agar was melted in a water bath at 100˚C for approximately 30 minutes and then kept at 56˚C in a heat cabinet until the test was initiated.

3.3.3 Isolation of bacteria

Fermented milk samples were collected from the starter culture activity test at the end of incubation and diluted in peptone water. Dilution series were performed for all fermented milk samples with a 100-fold dilution factor, from 10^{-2} to 10^{-6} (figure 2). Pre-melted calcium citrate agar (2 x 88 ml) was placed in 45˚C water bath. Peptone water (4 ml) was pipetted to the bacteria filtrate tube. One tube of bacteria filtrate and one tube of citrate solution were temperated in a water bath at 45˚C. Petri dishes were marked with sample-id, date and dilution. The bacteria filtrate and citrate solution were added to 88 ml calcium citrate agar followed by gentle mixing. The agar was then placed in a water bath at 45˚C for 5-10 minutes.

Dilutions 10^{-5}, 10^{-6} and 10^{-7} were pipetted to marked petri dishes as shown in figure 2. 250 µl x-gal solution/plate was pipetted to the petri dishes. A thin layer of calcium-citrate agar (with bacteria filtrate and citrate solution) was poured onto the plates followed by gentle mixing in order to achieve an even distribution of dilution, agar, and x-gal. After drying the media at room temperature for approximately 30 minutes, a second layer of calcium citrate agar (without bacteria filtrate and citrate solution) was poured on the top of the agar. After drying the second layer of media

for approximately 30 minutes, plates were incubated upside down at 25˚C for 72 hours.

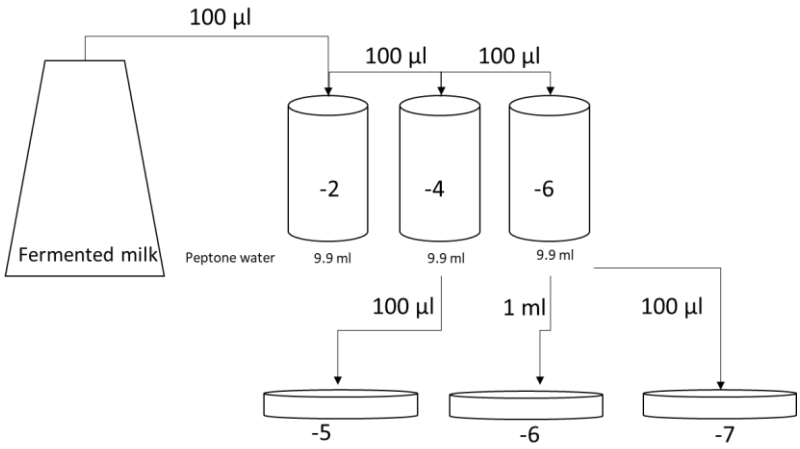

Figure 2. Illustration of dilution series performed in connection to agar plate preparation.

3.3.4 Examination of colonies

Colonies were examined under a light microscope. The number of colony forming units per ml (CFU/ml) was calculated for each bacterium. Bacteria morphology and characteristics are summarized in table 8. Colonies of *Leuconostoc spp.* and *Lactococcus lactis* spp. *lactis* biovar. diacetylactis appeared with surrounding zones. The zones surrounding *Leuconostoc* spp. colonies were blue and the zones surrounding colonies of *Lactococcus lactis* spp. *lactis* biovar. diacetylactis were clear. Colonies of acid producing *Lactococcus lactis* spp. *lactis* or *cremoris* appeared without surrounding zones (figure 3).

Figure 3. Colonies of *Leuconostoc* spp., *Lactococcus lactis* spp. *lactis* biovar. diacetylactis and *Lactococcus lactis* spp. *lactis/cremoris, respectively, cultured on x-gal-calcium citrate agar with added bacteria filtrate and citrate solution*

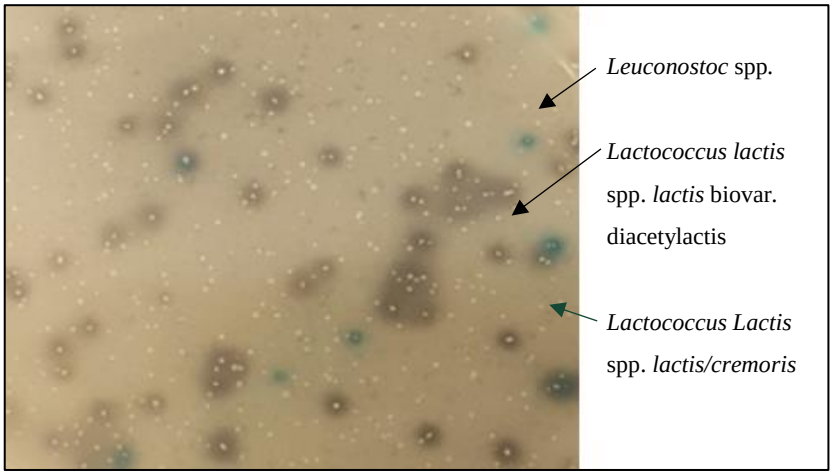

Table 8. Characteristics of colonies cultured on x-gal-calcium-citrate agar

			X-gal-calcium-citrate agar	
Species	Shape	Arrangement	Colour	Zone
Lactococcus lactis spp. lactis	Cocci[1]	Short chains[1]	White[2]	Absent[2]
Lactococcus lactis spp. cremoris	Cocci[1]	Short chains[1]	White[2]	Absent[2]
Lactococcus lactis spp. *lactis* biovar. diacetylactis	Cocci[1]	Short chains[1]	White[2]	Clear[2]
Leuconostoc mesenteroides spp.	Cocci[1]	Short chains[1]	Blue[2]	Absent or blue[2]
Leuconostoc lactis spp.	Cocci[1]	Short chains[1]	Blue[2]	Absent or blue[2]

[1] Madigan 2003; [2] Friedrich and Lenke 2006

3.4 Statistical analysis

One-way analysis of variance (ANOVA) was performed to investigate if antimicrobial drugs in milk had a significant effect on starter culture activity parameters (95 % confidence interval). Mean values and standard deviation were calculated for all replicates (n=3). Tukey pairwise comparison was used for pairwise investigation of differences in the number of different bacteria in the negative control sample and the replicates.

The statistical analyses were performed by Minitab 19.0 software (Minitab Inc., State College, PA, USA). Graphical illustrations were made using Microsoft® Excel® version 2203.

4. Results

4.1 Activity of starter cultures

4.1.1 Starter culture A

The fermentation of starter culture A is shown in figure 5 a-c. Penicillin G (4 ppb) and trimethoprim-sulfadiazine (100 ppb) showed no significant effects on the fermentation of starter culture A, while a trend (p = 0.093) was observed for 100 ppb oxytetracycline (table 9). The pH values at time 0 were between 6.60-6.79 for all replicates and the negative control. Difference in mean pH of the negative control and the replicates was statistically evaluated. At the end of fermentation, i.e. 15.5 h after the start of fermentation, the mean pH of samples with 100 ppb oxytetracycline was 8.1 % higher than the pH of negative control (figure 5b). Measurement values for figure 5 a-c are found in appendix I in table 1, 2 and 3.

Table 9. Effect of penicillin G (4 ppb), oxytetracycline (100 ppb) and trimethoprim-sulfadiazine (in combination 1:5, 100 ppb sulfadiazine), respectively, on fermentation (pH) of starter culture A. One-way ANOVA was performed to investigate the statistical significance of a reduction in fermentation by the antimicrobial drugs. P-value < 0.05 was considered significant

Antimicrobial drug	Significant effect (p-value)	Non-significant effect (p-value)
Penicillin G		(0.976)
Oxytetracycline		(0.093)
Trimethoprim-Sulfadiazine		(0.992)

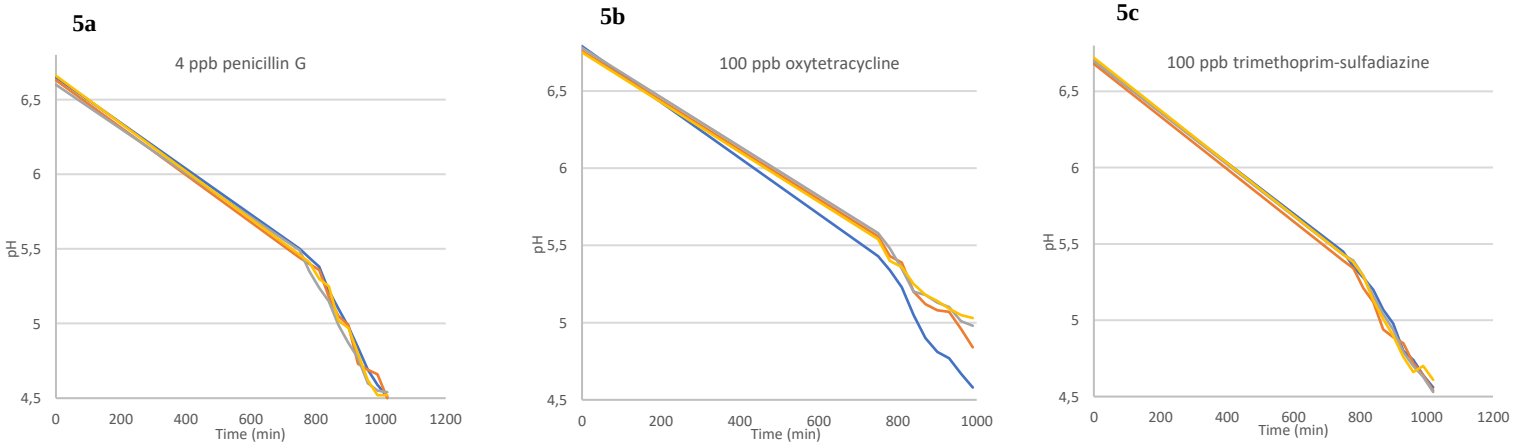

Figure 5a-c. Activity curve of starter culture A illustrating pH in the negative control sample and in the replicates 1, 2 and 3 with the respective antimicrobial drug added to the milk. Negative control = blue, replicate 1 = red, 2 = grey, 3 = yellow.

4.1.2 Starter culture B

The fermentation of starter culture B is shown in figure 6 a-c. Oxytetracycline (100 ppb) showed a significant effect (p=0.002) on the activity of starter culture B, while 4 ppb penicillin G and 100 ppb trimethoprim-sulfadiazine showed no significant effects (table 10). The pH-values at time 0 were between 6.59-6.67 for all replicates and the negative control. Difference in mean pH of the samples was statistically evaluated. At the end of fermentation, i.e. 16 h after the start of fermentation, the mean pH of samples with 100 ppb oxytetracycline was 4.4 % higher than the pH of the negative control (figure 6b). Measurement values for figure 6a-c are found in appendix I in table 4, 5 and 6.

Table 10. Effect of penicillin G (4 ppb), oxytetracycline (100 ppb) and trimethoprim-sulfadiazine (in combination 1:5, 100 ppb sulfadiazine), respectively, on fermentation (pH) of starter culture B. One-way ANOVA was performed to investigate the statistical significance of a reduction in fermentation by the antimicrobial drugs. P-value < 0.05 was considered significant

Antimicrobial drug	Significant effect (p-value)	Non-significant effect (p-value)
Penicillin G		(0.955)
Oxytetracycline	(0.002)	
Trimethoprim-Sulfadiazine		(0.971)

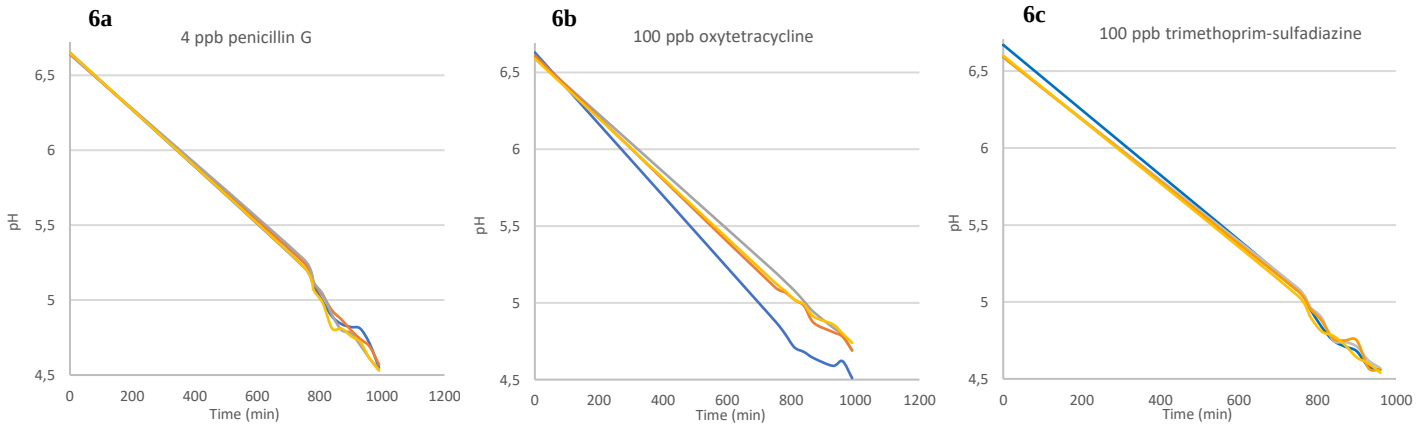

Figure 6a-c. Activity curve of starter culture B illustrating pH in the negative control sample and in the replicates 1, 2 and 3 with the respective antimicrobial drug added to the milk. Negative control = blue, replicate 1 = grey, replicate 2 = red, replicate 3 = yellow.

4.1.3 Starter culture C

The fermentation of starter culture C is shown in figure 7a–c. Oxytetracycline (100 ppb) showed a significant effect (p=0.000) on the activity of starter culture C, while 4 ppb penicillin G and 100 ppb trimethoprim-sulfadiazine showed no significant effects (table 11). The pH-values at time 0 were between 6.70-6.82 for all replicates and the negative control. Difference in mean pH of the samples was statistically evaluated. At the end of fermentation, i.e. 17.5 h after the start of fermentation, the mean pH of samples with 100 ppb oxytetracycline was 9.9 % higher than the pH of negative control (figure 7b). Measurement values for figure 7a-c can be found in appendix I in table 7, 8 and 9.

Table 11. Effect of penicillin G (4 ppb), oxytetracycline (100 ppb) and trimethoprim-sulfadiazine (in combination 1:5, 100 ppb sulfadiazine), respectively, on fermentation (pH) of starter culture C. One-way ANOVA was performed to investigate the statistical significance of a reduction in fermentation by the antimicrobial drugs. P-value < 0.05 was considered significant

Antimicrobial drug	Significant effect (p-value)	Non-significant effect (p-value)
Penicillin G		(0.999)
Oxytetracycline	(0.000)	
Trimethoprim-Sulfadiazine		(0.981)

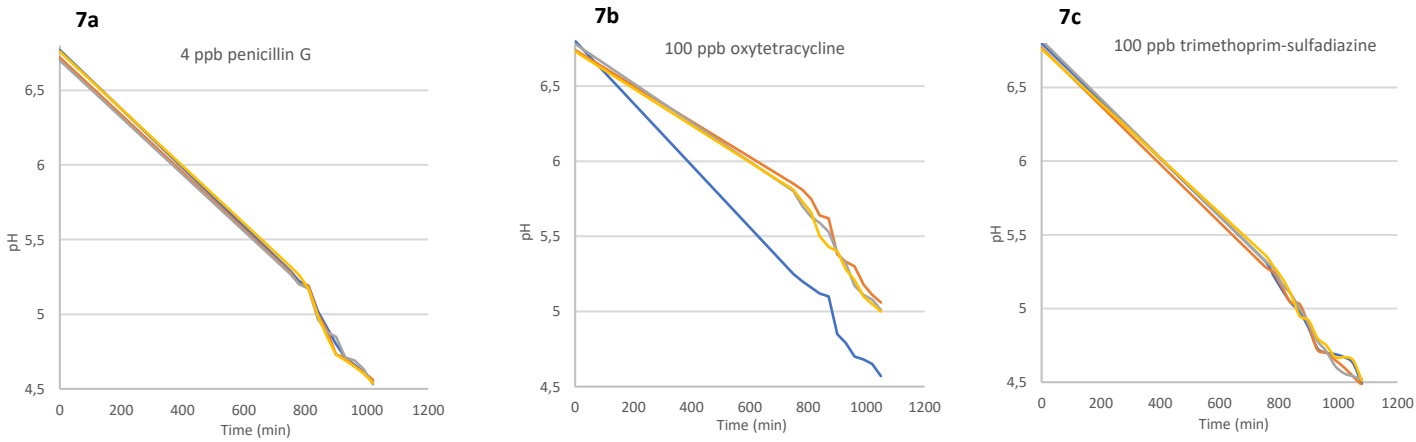

Figure 7a-c. Activity curve of starter culture C illustrating pH in the negative control sample and in the replicates 1, 2 and 3 with the respective antimicrobial drug added to the milk. Negative control = blue, rep. 1 = red, rep. 2 = grey, rep. 3 = yellow.

4.2 Composition of starter cultures

4.2.1 Starter culture A

In starter culture A, 4 ppb penicillin G, 100 ppb oxytetracycline and 100 ppb trimethoprim-sulfadiazine caused no significant inhibition of *Leuconostoc* spp., *Lactococcus lactis* spp. *lactis/cremoris* and *Lactococcus lactis* spp. *lactis* biovar. diacetylactis (table 12). None of the antimicrobial drugs had a significant effect on the number of any of the investigated bacteria in culture A. Due to the absence of observation values for *Leuconostoc* spp., statistical significance could not be evaluated for 100 ppb oxytetracycline and 100 ppb trimethoprim-sulfadiazine (table 13).

Table 12. Effect of penicillin G, oxytetracycline and trimethoprim-sulfadiazine on the number of colony forming units per ml in starter culture A. One-way ANOVA was performed to investigate the statistical significance of the inhibition of the starter bacteria by the antimicrobial drugs. Due to absence of observations, no p-values could be established for oxytetracycline and trimethoprim-sulfadiazine regarding *Leuconostoc* spp. A p-value < 0.05 was considered significant.

Antimicrobial drug	Bacteria	Significant (p-value)	Non-significant (p-value)
Penicillin G	*Leuconostoc* spp.		(0.184)
	Lactococcus lactis spp. *Lactis* biovar. diacetylactis		(0.199)
	Lactococcus lactis spp. *Lactis/cremoris*		(0.529)
Oxytetracycline	*Leuconostoc* spp.		
	Lactococcus lactis spp. *Lactis* biovar. diacetylactis		(0.147)
	Lactococcus lactis spp. *Lactis/cremoris*		(0.120)
Trimethoprim-sulfadiazine	*Leuconostoc* spp.		
	Lactococcus lactis spp. *Lactis* biovar. diacetylactis		(0.904)
	Lactococcus lactis spp. *Lactis/cremoris*		(0.506)

Table 13. Number of colony forming units per ml in the negative control and in the replicates with antimicrobial drugs. Average values and standard deviation (SD) are indicated. N=3 if not specified differently

	Leuconostoc spp.		*Lactococcus lactis* spp. *Lactis* biovar. diacetylactis		*Lactococcus lactis* spp. *Lactis/cremoris*	
	Negative control*	Replicates Average± SD	Negative control*	Replicates Average± SD	Negative control*	Replicates Average± SD
Penicillin G	4.0×10^5	$2.7 \times 10^5 \pm 5.8 \times 10^4$	3.0×10^7	$2.7 \times 10^7 \pm 1.5 \times 10^6$	2.3×10^8	$2.6 \times 10^8 \pm 3.1 \times 10^7$
Oxytetracycline	2.0×10^5	3.0×10^5*	3.4×10^7	$2.6 \times 10^6 \pm 3.0 \times 10^6$	2.6×10^8	$1.8 \times 10^8 \pm 2.6 \times 10^7$
Trimethoprim-sulfadiazine	0	*14.1* $\times 10^5 \pm 0$	4.5×10^7	$4.6 \times 10^7 \pm 8.5 \times 10^6$	2.6×10^8	$3.1 \times 10^8 \pm 5.0 \times 10^7$

*****Based on 1 measurement value.

4.2.2 Starter Culture B

No colonies of *Leuconostoc* spp. were detected in starter culture B. Oxytetracycline at 100 ppb caused a significant inhibition (p=0.022) of *Lactococcus lactis* spp. *Lactis* biovar. diacetylactis and a notable trend was observed for *Lactococcus lactis* spp. *Lactis/cremoris* (p=0.051). Penicillin G at 4 ppb did not result in a significant inhibition of *Lactococcus lactis* spp. *Lactis* biovar. diacetylactis, while a trend was seen for *Lactococcus lactis* spp. *Lactis/cremoris* (p=0.069). Trimethoprim-sulfadiazine at 100 ppb caused no significant inhibition of any of the investigated bacteria in culture B (table 14). Oxytetracycline (100 ppb) had significant effect on the number of CFU/ml of *Lactococcus lactis* spp. *Lactis* biovar. diacetylactis, while 4 ppb penicillin G and 100 ppb trimethoprim-sulfadiazine showed no effect (table 15). None of the antimicrobial drugs had significant effect on the number of *Lactococcus lactis* spp. *Lactis/cremoris.*

Table 14. Effect of penicillin G, oxytetracycline and trimethoprim-sulfadiazine on the number of colony forming units per ml in starter culture B. One-way ANOVA was performed to evaluate the statistical significance of the inhibition of the starter bacteria by the antimicrobial drugs. Due to absence of colonies, no p-values could be established for penicillin G, oxytetracycline or trimethoprim-sulfadiazine regarding *Leuconostoc* spp. A p-value < 0.05 was considered significant.

Antimicrobial drug	Bacteria	Significant (p-value)	Non-significant (p-value)
Penicillin G	*Leuconostoc* spp.*		
	Lactococcus lactis spp. *lactis* biovar. diacetylactis		(0.111)
	Lactococcus lactis spp. *lactis/cremoris*		(0.069)
Oxytetracycline	*Leuconostoc* spp.*		
	Lactococcus lactis spp. *lactis* biovar. diacetylactis	(0.022)	
	Lactococcus lactis spp. *lactis/cremoris*		(0.051)
Trimethoprim-sulfadiazine	*Leuconostoc* spp.*		
	Lactococcus lactis spp. *lactis* biovar. diacetylactis		(0.225)
	Lactococcus lactis spp. *lactis/cremoris*		(0.720)

*****Not detected in the starter culture

Table 15. Number of colony forming units per ml in the negative control and in the replicates with antimicrobial drugs. Average values and standard deviation (SD) are indicated. N=3 if not specified differently

	Leuconostoc spp.		*Lactococcus lactis* spp. *lactis* biovar. diacetylactis		*Lactococcus lactis* spp. *Lactis/cremoris*	
	Negative control*	Replicate Average± SD	Negative control*	Replicate Average± SD	Negative control*	Replicate Average± SD
Penicillin G	0	0	3.5×10^7	$2.5 \times 10^7 \pm 3.1 \times 10^6$	4.0×10^8	$3.1 \times 10^8 \pm 2.0 \times 10^7$
Oxytetracycline	0	0	$3.8 \times 10^{7\,A}$	$2.6 \times 10^{7\,B} \pm 1.5 \times 10^6$	2.3×10^8	$2.2 \times 10^8 \pm 2.5 \times 10^6$
Trimethoprim-sulfadiazine	0	0	4.5×10^7	$5.5 \times 10^7 \pm 5 \times 10^6$	2.2×10^8	$1.9 \times 10^8 \pm 6.4 \times 10^7$

*****Based on 1 measurement value. **A, B** If the superscripts associated to the numbers of CFU per ml in the negative control and the replicates with antimicrobial drugs are different for a bacterium, the difference between negative control and treatment is significant (p<0.05)

4.2.3 Starter culture C

In starter culture C, 100 ppb oxytetracycline caused significant inhibition of *Leuconostoc* spp. (p=0.015), *Lactococcus lactis* spp. *lactis* biovar. diacetylactis (p=0.003) and *Lactococcus lactis* spp. *Lactis/cremoris* (0.032). Penicillin G at 4 ppb caused significant inhibition of *Lactococcus lactis* spp. *Lactis/cremoris* (p=0.049), while there was no significant inhibition of *Leuconostoc* spp. and *Lactococcus lactis* spp. *lactis* biovar. diacetylactis. Trimethoprim-sulfadiazine at 100 ppb caused no significant inhibition of any of the investigated bacteria in culture C (table 16). Oxytetracycline at 100 ppb had a significant effect on the number of colony forming units of *Leuconostoc* spp., *Lactococcus lactis* spp. *lactis* biovar. diacetylactis and *Lactococcus lactis* spp. *lactis/cremoris* (table 17). Penicillin G at 4 ppb only had a significant effect on the number of CFU/ml of *Lactococcus lactis* spp. *lactis/cremoris* whereas 100 ppb trimethoprim-sulfadiazine had no significant effect on the number of any of the investigated bacteria in culture C (table 17).

Table 16. Effect of penicillin G, oxytetracycline and trimethoprim-sulfadiazine on the number of colony forming units per ml in starter culture C. One-way ANOVA was performed to evaluate the statistical significance of the inhibition of the starter bacteria by the antimicrobial drugs. A p-value < 0.05 was considered significant.

Antimicrobial drug	Bacteria	Significant (p-value)	Non-significant (p-value)
Penicillin G	*Leuconostoc* spp.		(0.635)
	Lactococcus lactis spp. *lactis* biovar. diacetylactis		(0.858)
	Lactococcus lactis spp. *lactis/cremoris*	(0.049)	
Oxytetracycline	*Leuconostoc* spp.	(0.015)	
	Lactococcus lactis spp. *lactis* biovar. diacetylactis	(0.003)	
	Lactococcus lactis spp. *lactis/cremoris*	(0.032)	
Trimethoprim-sulfadiazine	*Leuconostoc* spp.		(0.397)
	Lactococcus lactis spp. *lactis* biovar. diacetylactis		(0.136)
	Lactococcus lactis spp. *lactis/cremoris*		(0.525)

Table 17. Number of colony forming units per ml in the negative control and in the replicates with antimicrobial drugs. Average values and standard deviation (SD) are indicated. N=3 if not specified differently

	Leuconostoc spp.		*Lactococcus lactis* spp. *lactis* biovar. diacetylactis		*Lactococcus lactis* spp. *Lactis/cremoris*	
	Negative control*	Replicate Average± SD	Negative control*	Replicate Average± SD	Negative control*	Replicate Average± SD
Penicillin G	2.0×10^7	$2.1 \times 10^7 \pm 2.1 \times 10^6$	8.5×10^7	$8.4 \times 10^7 \pm 5.7 \times 10^6$	$5.7 \times 10^{8\,A}$	$5.2 \times 10^{8\,B} \pm 1.0 \times 10^7$
Oxytetracycline	2.8×10^{7A}	$2.3 \times 10^{7B} \pm 5.8 \times 10^5$	$1.2 \times 10^{8\,A}$	$8.0 \times 10^{7\,B} \pm 2 \times 10^6$	$4.6 \times 10^{8\,A}$	$3.6 \times 10^{8\,B} \pm 1.5 \times 10^7$
Trimethoprim-sulfadiazine	1.6×10^7	$2.0 \times 10^7 \pm 3.5 \times 10^6$	7.9×10^7	$6.5 \times 10^7 \pm 5 \times 10^6$	4.6×10^8	$4.3 \times 10^8 \pm 3.8 \times 10^7$

*****Based on 1 measurement value. **A, B** If the superscripts associated to the numbers of CFU per ml in the negative control and the replicates with antimicrobial drugs are different for a bacterium, the difference between negative control and treatment is significant (p<0.05)

5. Discussion

5.1 Starter culture activity

In this study, antimicrobial drug residues´ effect on starter culture activity was investigated by measuring pH during fermentation. Similarities in the appearance of the pH-curves indicated that the investigated starter cultures followed approximately the same fermentation pattern.

Oxytetracycline at 100 ppb had a highly significant effect on the activity of starter culture B and C while the effect was less clear for starter culture A. Penicillin G at 4 ppb and 100 ppb trimethoprim-sulfadiazine showed no significant effects on the activity of the investigated starter cultures. The differences between the antimicrobial drugs were illustrated by the pH-curves. pH-curves of 100 ppb oxytetracycline were uniformly positioned above the curve of the negative control while pH-curves of 4 ppb penicillin G and 100 ppb trimethoprim-sulfadiazine were more or less identical to the negative control curve. pH values measured at time 0 were not included in the statistical analysis to receive a more representative result. An effect of the antimicrobial drugs was not expected to be observed at time 0 due to the initial lag phase of the starter culture bacteria.

The antimicrobial drugs included in the study were expected to have different effects on the starter cultures due to differences in their mechanism of action. Oxytetracycline is a broad-spectrum antimicrobial drug that affect both gram-positive and negative bacteria. In addition, oxytetracycline was previously reported to have the capacity to affect *Lactococcus* spp. and *Leuconostoc* spp. at concentrations below MRL (Katla et al 2000).

Trimethoprim and sulfadiazine were not expected to affect investigated starter cultures at concentrations corresponding to the MRL. Both trimethoprim and sulfadiazine target folic acid metabolism. Since LAB rely on folic acid from external sources, the bacteria are less susceptible to antimicrobial drugs that target folic acid metabolism.

Each antimicrobial drug was investigated once per starter culture. All tests included triplicates of antimicrobial drug samples and one negative control sample. To further confirm the results of the study, it would be necessary to perform additional experiments.

Due to limited access to instruments for pH measurements in this study, pH was measured manually at fixed occasions. Automated and continuous measurement of pH would have improved the method, resulting in a higher number of measuring values throughout the fermentation process. By that, more reliable pH-curves could be produced. Initially, a substantial part of the work was devoted to find an optimal way to perform the starter culture activity test. At first, the fermentation was initiated at 8 am in the morning, resulting in a high number of measuring points during the bacterial lag phase. In addition, the end point of fermentation was reached during the night when pH was not measured. That made the first protocol unsuitable for the study. In the revised protocol, fermentation was instead initiated at 8 pm in the evening, with the result that the fermentation end point was reached the following day. This made it possible to measure pH during the interval when end-pH was approaching. By that, it was possible to receive a first indication whether penicillin G, oxytetracycline and trimethoprim-sulfadiazine had an impact on the fermentation of the investigated starter cultures at concentrations corresponding to their respective MRLs. In addition, it could be ensured that the collection of fermented milk samples for cultivation occurred at the same point of the fermentation process in all experiments. This could be of relevance since bacteria possess different activities during different stages of the fermentation process (Walstra et al 2006).

5.2 Starter culture composition

In this study, starter culture bacteria in fermented milk samples were cultivated on x-gal calcium citrate agar to investigate how the relative proportions of acid- and aroma producing bacteria was affected by antimicrobial residues in the milk. Cultivation of starter culture bacteria at the end of fermentation was a complement to the activity test where the effect of antimicrobial drugs on acid production during fermentation was investigated.

In the study, 4 ppb penicillin G had no significant effect on *Leuconostoc* spp., *Lactococcus lactis* spp. *lactis* biovar. diacetylactis or *Lactococcus lactis* spp. *lactis/cremoris* in starter culture A or B. In starter culture C, however, 4 ppb penicillin G had a significant effect on *Lactococcus lactis* spp. *lactis/cremoris*. An explanation to the difference in effect between the starter cultures could be that the starter cultures had different strain composition. In addition, differences in the experimental procedure could potentially have had an effect on the result since the starter cultures were not investigated in the same experiment. Important parameters are for example antimicrobial drug concentration, fermentation temperature and fermentation length.

The results were in accordance with findings of Katla et al (2000), who showed that 94 ppb penicillin G was required to cause 50 % inhibition of *Lactococcus* spp. whereas 380 ppb penicillin G was required to cause 50 % inhibition of *Leuconostoc* spp. However, the minimum inhibitory concentration value (MIC) was found to be in range of 2-300 ppb for *Lactococcus* spp. and 16-200 ppb for *Leuconostoc* spp. (Katla et al 2000).

Oxytetracycline at 100 ppb showed no significant effect on bacteria in starter culture A, while it showed a significant effect on *Lactococcus lactis* spp. *lactis* biovar. diacetylactis in starter culture B. In starter culture C, 100 ppb oxytetracycline showed a significant effect on all bacteria. Due to oxytetracycline´s capacity to inhibit a broad spectrum of bacteria, an effect on LAB was expected. In a study by Katla et al (2000), 250 ppb tetracycline caused 50 % inhibition of *Lactococcus* spp. while 1000 ppb tetracycline caused 50 % inhibition of *Leuconostoc* spp. Similar to penicillin G, the MIC values were in large ranges. 64-8000 ppb tetracycline caused inhibition of *Lactococcus* spp. whereas 380-6000 ppb tetracycline caused inhibition of *Leuconostoc* spp.

In contrast to 100 ppb oxytetracycline, 100 ppb trimethoprim-sulfadiazine showed no significant effect on the bacteria in starter culture A, B or C. The result was expected due to the mechanism of action of trimethoprim and sulfadiazine. Both trimethoprim and sulfadiazine targets folic acid metabolism in bacteria and since LAB receive folic acid from external sources, the sensitivity to antimicrobial drugs which target this mechanism of action is low (Sykes and Papich 2014; Madigan 2003). The low sensitivity of *Lactococcus* spp. and *Leuconostoc* spp. to trimethoprim and sulfadiazine was also showed by Katla et al (2000). For sulfadiazine, a concentration higher than 256 ppb was required to cause 50 % inhibition of *Lactococcus* spp. and *Leuconostoc* spp, while a concentration higher than 32 ppb was required for trimethoprim to cause 50 % inhibition of *Lactococcus* spp. and *Leuconostoc* spp. The MIC value of both sulfadiazine and trimethoprim were above the breakpoint of resistance for *Lactococcus* spp. and *Leuconostoc* spp. (Katla et al 2000).

According to manufacturer´s product information, all starter cultures in the study consisted of the same bacteria species. However, strain composition was unknown. Cultivation of starter culture bacteria made it possible to receive further information regarding starter culture composition. *Lactococcus lactis* spp. *lactis* and/or *cremoris* were most abundant in all starter cultures, followed by *Lactococcus lactis* spp. *lactis* biovar. diacetylactis. *Leuconostoc* spp. were the least abundant bacteria in all starter cultures. In starter culture A, all samples contained less than 5×10^5 CFU/ml of *Leuconostoc* spp. *Leuconostoc* spp. was not detected at all in starter culture B at the experiment´s lowest detectable level, 10^5 CFU/ml, while starter culture C had the highest level of *Leuconostoc* spp (10^7 CFU/ms).

Leuconostoc spp. often constitutes a minor part of mesophilic starter cultures (Mathot et al 1994). Due to the similarities in cell morphology between the starter culture bacteria, it is not possible to determine starter culture composition by direct examination using microscope. Instead, enumeration is preferably performed using a selective media (Mathot et al 1994).

In this study, x-gal calcium citrate agar was used to differentiate and enumerate *Leuconostoc* spp., *Lactococcus lactis* spp. *lactis* biovar. diacetylactis, and *Lactococcus lactis* spp. *lactis/cremoris* in three mesophilic starter cultures used for cheese production. The selectivity of x-gal calcium agar relies on the difference in citrate fermentation in aroma- and acid producing bacteria and the difference in x-gal hydrolysation within aroma producing bacteria (Mathot et al 1994).

Initially, 10^{-6} and 10^{-7} dilutions were cultivated. The dilutions generated a suitable number of colony forming units per plate of *Lactococcus lactis* spp. *lactis/cremoris* and *Lactococcus lactis* spp. *lactis* biovar. diacetylactis, while the number of *Leuconostoc* spp. colonies was considered too low for reliable counting. Subsequently, 10^{-5} dilution was included in the protocol to investigate if the number of colony forming units of *Leuconostoc* spp. per plate to increase the reliability of the method. However, the numbers of *Leuconostoc* spp. still remained low. Overgrowth of *Lactococcus lactis* spp. *lactis/cremoris* and *Lactococcus lactis* spp. *lactis* biovar. diacetylactis made it difficult to distinguish colonies of *Leuconostoc* spp. To prevent overgrowth of *Lactococcus lactis* spp. *lactis/cremoris* and *Lactococcus lactis* spp. *lactis* biovar. diacetylactis, the 10^{-5} dilutions were examined after both 48 and 72 hours. However, incubation for 72 hours was required for growth of *Leuconostoc* spp. The numbers of CFU/ml for *Leuconostoc* spp. presented in this study are thus often below 30 colony forming units per plate. To conclude, attempts to create conditions to assure reliable enumeration of *Leuconostoc* spp. were not fully successful on these selective plates.

It has been suggested in previous studies that using a single selective media might be unsuitable if the proportion of *Leuconostoc* spp. is too low (Mathot et al 1994). This indicates that other selection agents, not used in this study, might result in higher numbers of *Leuconostoc* spp. Another selective agent in the agar was citrate. Due to differences in citrate fermentation in aroma- and acid producing bacteria, *Lactococcus lactis* spp. *lactis/cremoris* could be distinguished from *Leuconostoc* spp. and *Lactococcus lactis* spp. *lactis* biovar. diacetylactis. However, other bacteria that potentially could be present in thes milk, such as *Lactobacillus* spp., also ferment citrate (Mathot et al 1994). To ensure that colonies surrounded with zones were *Leuconostoc* spp. or *Lactococcus lactis* spp. *lactis* biovar. diacetylactis, it was thus necessary to examine colonies under a microscope. In addition, not all strains of *Leuconostoc* spp. ferment citrate (Hemme & Foucaud-Scheunemann 2004). This could potentially decrease the reliability of the method since it was not possible to ensure that *Leuconostoc* spp. present in the starter

cultures were capable to ferment citrate. In addition, it was not specified what *Leuconostoc* spp. that were included in the starter cultures.

5.3 Relevance for dairy production

The selection of antimicrobial drugs for this study was based on their use in dairy cattle in Sweden. β-lactams are by far the most frequently prescribed type of antimicrobial drugs in dairy production in 2001-2020 followed by tetracyclines, trimethoprim and sulphonamides (Växa 2020).

Investigated concentrations in the study were corresponding to the respective MRL value in milk of the selected antimicrobials. Oxytetracycline had a significant effect on acid- and aroma producing bacteria at the MRL value of 100 ppb. Penicillin G had no significant effect on starter culture activity at MRL levels, but significant inhibition of acid producing bacteria was observed in the starter culture composition test. Trimethoprim-sulfadiazine had neither a significant effect on starter culture activity nor composition.

The occurrence of antimicrobial drug residues in milk at concentrations above MRL is probably not frequent in Sweden. This, because detection limits of the available tests for control of antimicrobial drugs typically correspond to MRLs. In addition, the prescription of antimicrobial drugs in Sweden is among the lowest within EU (European Medicines Agency 2020). B-lactams, including penicillin G, were prescribed to 10.81 per 100 milking cows in Sweden 2020. For tetracyclines, which includes oxytetracycline, the number of prescriptions was 0.65 per 100 milking cows while the prescription incidence for sulphonamides and trimethoprim was 0.56 per 100 milking cows (Växa 2020).

It remains of relevance for dairy industry to keep updated on how the prescription of antimicrobial drugs in a geographical area develops during time. Increased treatment frequency of other illnesses than mastitis could potentially increase the prescription incidence of other antimicrobial drugs than penicillin G. Due to oxytetracycline´s potential to inhibit mesophilic starter culture bacteria at concentrations corresponding to the MRL, tests that also include detection of this antimicrobial drug could be of relevance for the dairy industry.

6. Conclusion

Due to MRL regulations of and low prescription incidences, the occurrence of antimicrobial drug residues in milk is not a pronounced concern in dairy industry in Sweden. However, residues of antimicrobial drugs might affect starter culture bacteria at concentrations that are not detected by the commercial tests that are used for control. Decreased starter culture activity might impair acidification, and changes in starter culture composition might affect the aroma formation during cheese production, resulting in lower yield as well as impaired product quality. To ensure that residues of antimicrobials that might interfere with starter culture bacteria are not present in milk intended for cheese production, it could be of relevance to implement tests on dairy silo level that detect a wider spectrum of antimicrobial residues. This is supported by the current study where oxytetracycline had significant effects on both acid and aroma producing bacteria at concentrations corresponding to MRL. However, further work is necessary to confirm the results of this study. Investigations of other concentrations could be of interest to further establish the limit for inhibition of the mesophilic starter culture bacteria. In addition, it would be of relevance to investigate how other starter cultures of interest for production of other types of cheese or fermented milk products are affected by residues of antimicrobial drugs in milk.

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Popular science summary

Have you ever wondered what impacts cheese quality? Aroma, flavour and texture of cheese depend on a number of parameters such as raw milk characteristics, manufacture and composition of microflora. Successful cheese manufacture commonly requires an active and balanced microflora. The individual microflora of a cheesemaking plant, i.e., the houseflora, contribute to unique cheese characteristics. However, lactic acid bacteria are commonly also added as standardised starter cultures. Addition of cheese starter culture increases milk acidity due to bacterial conversion of lactose to lactic acid. Low pH facilitates milk coagulation and prevents growth of pathogens or unwanted spoilage organisms. Starter culture bacteria are in addition essential for the development of cheese flavour and texture during cheese maturation.

Important parameters that affect activity of cheese starter cultures are milk temperature, pH and presence of antimicrobial drug residues. Antimicrobial drugs are used to treat food producing animals for infections, and sometimes residues may be transferred to the milk. Use of antimicrobial drugs in dairy production is globally followed. Extensive use of antimicrobial drugs for preventive and curative treatment increases the occurrence of antibiotic resistant bacteria and increase the risk for antimicrobial residues in dairy products. This can potentially be hazardous to human health. The most common disease that leads to antibiotic treatment of dairy cows is mastitis. Antimicrobial drugs in dairy production include β-lactams, tetracyclines, sulphonamides and trimethoprim.

Low use of antimicrobial drugs in dairy production in Sweden and regulations regarding maximum levels of antimicrobial drugs in milk likely contribute to keeping antimicrobial residues on a low level. However, antimicrobial residues can interfere with starter culture bacteria even at low concentrations which go undetected by some methods. That could decrease the activity of starter cultures in cheese production, which in turn could impair cheese quality, result in a lower cheese yield and cause economical losses for the dairy. One question of relevance is if antimicrobial drugs have the capacity to impair starter culture activity at levels below the detection limit of commercial tests that are used in the dairy industry today. If that is the case, will cheese quality be affected?

The aim of this study was to investigate how penicillin G, oxytetracycline and trimethoprim-sulfadiazine affect the bacteria in commercial mesophilic starter cultures at concentrations corresponding to their respective maximum residue level. The result of the study showed that the effect to a large extent is dependent on the antimicrobial drug type. The antimicrobial drug oxytetracycline showed significant effects on both acid and aroma producing bacteria at concentrations corresponding to the maximum residue level. Penicillin G only showed a few significant effects on starter culture activity while trimethoprim-sulfadiazine showed no significant effects on starter culture activity. Based on the result of this study, it might be of relevance for the dairy industry to implement antibiotic tests that includes detection of a broader spectrum of antimicrobial drugs. However, further studies are necessary to confirm the results.

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Appendix I Activity data

Table 1. Values registered for starter culture A during incubation in containing 4 ppb penicillin G.

Time (min.)	Negative control		Replicate 1		Replicate 2		Replicate 3	
	pH	Temp. C˚	pH	Temp. C˚	pH	Temp. C˚	pH	Temp. C˚
0	6.65	19.9	6.63	19.8	6.6	20	6.66	19.9
750	5.5	20.4	5.44	20.4	5.49	20.6	5.46	20.6
780	5.44	20.4	5.4	20.5	5.35	20.5	5.41	20.6
810	5.38	20.5	5.36	20.4	5.24	20.5	5.3	20.5
840	5.22	20.5	5.18	20.4	5.15	20.5	5.25	20.5
870	5.1	20.5	5.05	20.4	4.99	20.5	5.02	20.4
900	4.98	20.4	4.99	20.5	4.87	20.5	4.97	20.5
930	4.84	20.5	4.73	20.5	4.77	20.5	4.8	20.5
960	4.69	20.5	4.69	20.5	4.6	20.5	4.62	20.5
990	4.59	20.5	4.66	20.5	4.55	20.5	4.52	20.5
1020	4.51	20.4	4.5	20.3	4.54	20.5	4.52	20.4

Table 2. Values registered for starter culture A during incubation in milk containing 100 ppb oxytetracycline

Time (min.)	Negative control		Replicate 1		Replicate 2		Replicate 3	
	pH	Temp. C˚	pH	Temp. C˚	pH	Temp. C˚	pH	Temp. C˚
0	6.79	20.9	6.76	20.9	6.78	20.8	6.75	20.8
750	5.43	20.6	5.56	20.7	5.58	20.5	5.54	20.6
780	5.34	20.6	5.43	20.4	5.48	20.6	5.4	20.4
810	5.23	20.4	5.39	20.5	5.35	20.4	5.36	20.5
840	5.05	20.5	5.2	20.5	5.2	20.4	5.25	20.5
870	4.9	20.4	5.12	20.5	5.18	20.4	5.18	20.5
900	4.81	20.4	5.08	20.5	5.13	20.4	5.14	20.5
930	4.77	20.4	5.07	20.4	5.1	20.4	5.09	20.5
960	4.67	20.4	4.96	20.4	5.01	20.4	5.05	20.5
990	4.58	20.5	4.84	20.4	4.98	20.5	5.03	20.4

Table 3. Values registered for starter culture A during incubation in milk containing 100 ppb trimethoprim-sulfadiazine

Time (min.)	Negative control		Replicate 1		Replicate 2		Replicate 3	
	pH	Temp. C˚	pH	Temp. C˚	pH	Temp. C˚	pH	Temp. C˚
0	6.7	21	6.68	20.9	6.7	20.9	6.72	20.8
750	5.45	20.6	5.39	20.5	5.43	20.6	5.43	20.6
780	5.35	20.5	5.34	20.6	5.39	20.4	5.38	20.5
810	5.28	20.4	5.21	20.5	5.29	20.4	5.29	20.5
840	5.2	20.5	5.12	20.5	5.16	20.4	5.13	20.5
870	5.07	20.4	4.94	20.5	5.04	20.4	5.01	20.4
900	4.98	20.4	4.89	20.5	4.94	20.5	4.9	20.4
930	4.8	20.5	4.85	20.5	4.79	20.5	4.76	20.4
960	4.74	20.5	4.72	20.5	4.7	20.5	4.66	20.4
990	4.64	20.5	4.64	20.5	4.63	20.5	4.7	20.4
1020	4.56	20.3	4.54	20.4	4.53	20.4	4.61	20.5

Table 4. Values registered for starter culture B during incubation in milk containing 4 ppb penicillin

Time (min.)	Negative control		Replicate 1		Replicate 2		Replicate 3	
	pH	Temp. C˚	pH	Temp. C˚	pH	Temp. C˚	pH	Temp. C˚
0	6.65	20.8	6.64	20.7	6.64	20.6	6.65	20.6
750	5.22	20.6	5.25	20.5	5.27	20.5	5.22	20.5
780	5.10	20.5	5.12	20.4	5.12	20.4	5.07	20.5
810	4.99	20.3	5.03	20.3	5.04	20.5	4.98	20.3
840	4.89	20.5	4.93	20.5	4.9	20.5	4.81	20.4
870	4.84	20.5	4.87	20.5	4.8	20.4	4.81	20.4
900	4.82	20.5	4.8	20.4	4.78	20.5	4.76	20.5
930	4.81	20.4	4.74	20.4	4.7	20.4	4.72	20.4
960	4.71	20.4	4.69	20.4	4.61	20.4	4.61	20.4
990	4.55	20.4	4.57	20.4	4.54	20.4	4.53	20.4

G

Table 5. Values registered for starter culture B during incubation in milk containing 100 ppb oxytetracycline

Time (min.)	Negative control		Replicate 1		Replicate 2		Replicate 3	
	pH	Temp. C˚	pH	Temp. C˚	pH	Temp. C˚	pH	Temp. C˚
0	6.63	21.1	6.6	21.1	6.61	21.1	6.59	21.2
750	4.88	20.7	5.2	20.5	5.1	20.6	5.13	20.6
780	4.80	20.7	5.14	20.5	5.07	20.6	5.08	20.6
810	4.71	20.6	5.08	20.5	5.02	20.6	5.02	20.6
840	4.68	20.6	5.01	20.6	4.98	20.6	4.99	20.5
870	4.64	20.5	4.94	20.5	4.87	20.6	4.91	20.5
900	4.59	20.5	4.84	20.5	4.81	20.6	4.86	20.6
930	4.62	20.4	4.79	20.5	4.78	20.6	4.8	20.5
960	4.51	20.4	4.69	20.6	4.69	20.6	4.74	20.3

Table 6. Values registered for starter culture B during incubation in milk containing 100 ppb trimethoprim-sulfadiazine

Time (min.)	Negative control		Replicate 1		Replicate 2		Replicate 3	
	pH	Temp. C˚	pH	Temp. C˚	pH	Temp. C˚	pH	Temp. C˚
0	6.67	20	6.59	20	6.59	20.1	6.6	20.1
750	5.08	20.6	5.09	20.5	5.07	20.5	5.04	20.6
780	4.96	20.5	4.97	20.4	4.96	20.5	4.91	20.5
810	4.84	20.5	4.9	20.4	4.88	20.5	4.81	20.4
840	4.75	20.6	4.75	20.5	4.76	20.5	4.78	20.5
870	4.71	20.5	4.74	20.5	4.75	20.5	4.72	20.5
900	4.68	20.5	4.71	20.3	4.75	20.4	4.64	20.5
930	4.58	20.5	4.62	20.4	4.57	20.5	4.6	20.4
960	4.56	20.4	4.57	20.5	4.56	20.3	4.54	20.5

Table 7. Values registered for starter culture C during incubation in milk containing 4 ppb penicillin G

Time (min.)	Negative control		Replicate 1		Replicate 2		Replicate 3	
	pH	Temp. C˚	pH	Temp. C˚	pH	Temp. C˚	pH	Temp. C˚
0	6.77	21	6.72	21	6.7	21	6.76	21
750	5.29	20.4	5.28	20.3	5.27	20.5	5.32	20.4
780	5.22	20.4	5.2	20.4	5.2	20.5	5.26	20.5
810	5.19	20.5	5.19	20.5	5.17	20.4	5.17	20.4
840	5.02	20.5	5	20.5	4.97	20.5	4.99	20.5
870	4.91	20.5	4.89	20.5	4.88	20.5	4.85	20.4
900	4.8	20.5	4.73	20.4	4.85	20.5	4.73	20.5
930	4.71	20.5	4.71	20.5	4.71	20.5	4.69	20.4
960	4.66	20.5	4.65	20.5	4.69	20.5	4.65	20.4
990	4.61	20.6	4.61	20.5	4.63	20.6	4.6	20.5
1020	4.55	20.6	4.56	20.5	4.53	20.6	4.54	20.5

Table 8. Values registered for starter culture C during incubation in milk containing 100 ppb oxytetracycline

Time (min.)	Negative control		Replicate 1		Replicate 2		Replicate 3	
	pH	Temp. C˚	pH	Temp. C˚	pH	Temp. C˚	pH	Temp. C˚
0	6.8	20.8	6.74	20.7	6.78	20.6	6.73	20.6
750	5.25	20.4	5.85	20.4	5.8	20.4	5.81	20.4
780	5.20	20.4	5.81	20.4	5.7	20.4	5.73	20.4
810	5.16	20.4	5.75	20.3	5.63	20.4	5.66	20.4
840	5.12	20.4	5.64	20.3	5.59	20.4	5.5	20.4
870	5.1	20.3	5.62	20.3	5.53	20.3	5.43	20.3
900	4.85	20.5	5.38	20.3	5.39	20.3	5.4	20.3
930	4.79	20.5	5.33	20.3	5.32	20.1	5.28	20.3
960	4.7	20.5	5.3	20.3	5.17	20.3	5.21	20.3
990	4.68	20.5	5.18	20.3	5.11	20.5	5.1	20.5
1020	4.65	20.4	5.11	20.4	5.08	20.4	5.05	20.4
1050	4.57	20.4	5.06	20.5	5.01	20.5	5	20.1

Table 9. Values registered for starter culture C during incubation in milk containing 100 ppb trimethoprim-sulfadiazine

Time (min.)	Negative control		Replicate 1		Replicate 2		Replicate 3	
	pH	Temp. C˚	pH	Temp. C˚	pH	Temp. C˚	pH	Temp. C˚
0	6.8	20.6	6.77	20.6	6.82	20.5	6.76	20.5
750	5.33	20.4	5.29	20.5	5.33	20.5	5.37	20.5
780	5.23	20.4	5.25	20.5	5.28	20.5	5.29	20.5
810	5.13	20.4	5.15	20.5	5.17	20.5	5.21	20.5
840	5.04	20.5	5.04	20.5	5.1	20.4	5.1	20.5
870	4.98	20.5	5.03	20.4	5	20.5	4.95	20.5
900	4.87	20.5	4.89	20.5	4.9	20.5	4.92	20.5
930	4.73	20.4	4.72	20.5	4.78	20.5	4.8	20.5
960	4.7	20.4	4.7	20.4	4.71	20.5	4.75	20.5
990	4.69	20.5	4.65	20.4	4.61	20.5	4.67	20.4
1020	4.67	20.5	4.6	20.4	4.56	20.5	4.67	20.4
1050	4.63	20.4	4.54	20.4	4.54	20.5	4.65	20.4
1080	4.49	20.3	4.48	20.3	4.52	20.2	4.51	20.4

Appendix II Cultivation data

Table 10. Colony forming units per ml (CFU/ml) in negative control and replicates 1, 2 and 3 of starter culture A inoculated in milk containing 4 ppb penicillin G

Bacteria	CFU/ml			
	Negative control	Replicate 1	Replicate 2	Replicate 3
Leuconostoc spp.	400 000	300 000	300 000	200 000
Lactococcus lactis spp. *lactis* biovar. diacetylactis	30 000 000	28 000 000	25 000 000	27 000 000
Lactococcus lactis spp. *lactis/cremoris*	230 000 000	250 000 000	290 000 000	230 000 000

Table 11. Colony forming units per ml (CFU/ml) in negative control and replicates 1, 2 and 3 of starter culture A inoculated in milk containing 100 ppb oxytetracycline

Bacteria	CFU/ml			
	Negative control	Replicate 1	Replicate 2	Replicate 3
Leuconostoc spp.	200 000	0	0	300 000
Lactococcus lactis spp. *lactis* biovar. diacetylactis	34 000 000	23 000 000	26 000 000	29 000 000
Lactococcus lactis spp. *lactis/cremoris*	260 000 000	150 000 000	200 000 000	190 000 000

Table 12. Colony forming units per ml (CFU/ml) in negative control and replicates 1, 2 and 3 of starter culture A inoculated in milk containing 100 ppb trimethoprim-sulfadiazine

Bacteria	CFU/ml			
	Negative control	Replicate 1	Replicate 2	Replicate 3
Leuconostoc spp.	0	0	0	0
Lactococcus lactis spp. *lactis* biovar. diacetylactis	45 000 000	43 000 000	40 000 000	56 000 000
Lactococcus lactis spp. *lactis/cremoris*	260 000 000	300 000 000	360 000 000	260 000 000

Table 13. Colony forming units per ml (CFU/ml) in negative control and replicates 1, 2 and 3 of starter culture B inoculated in milk containing 4 ppb penicillin G

Bacteria	CFU/ml			
	Negative control	Replicate 1	Replicate 2	Replicate 3
Leuconostoc spp.	0	0	0	0
Lactococcus lactis spp. *lactis* biovar. diacetylactis	35 000 000	28 000 000	22 000 000	26 000 000
Lactococcus lactis spp. *lactis/cremoris*	400 000 000	330 000 000	320 000 000	290 000 000

Table 14. Colony forming units per ml (CFU/ml) in negative control and replicates 1, 2 and 3 of starter culture B inoculated in milk containing 100 ppb oxytetracycline

Bacteria	CFU/ml			
	Negative control	Replicate 1	Replicate 2	Replicate 3
Leuconostoc spp.	0	0	0	0
Lactococcus lactis spp. *lactis* biovar. diacetylactis	38 000 000	25 000 000	28 000 000	26 000 000
Lactococcus lactis spp. *lactis/cremoris*	230 000 000	218 000 000	220 000 000	215 000 000

Table 15. Colony forming units per ml (CFU/ml) in negative control and replicates 1, 2 and 3 of starter culture B inoculated in milk containing 100 ppb trimethoprim-sulfadiazine

Bacteria	CFU/ml			
	Negative control	Replicate 1	Replicate 2	Replicate 3
Leuconostoc spp.	0	0	0	0
Lactococcus lactis spp. *lactis* biovar. diacetylactis	45 000 000	60 000 000	50 000 000	55 000 000
Lactococcus lactis spp. *lactis/cremoris*	221 000 000	225 000 000	116 000 000	230 000 000

Table 16. Colony forming units per ml (CFU/ml) in negative control and replicates 1, 2 and 3 of starter culture C inoculated in milk containing 4 ppb penicillin G

Bacteria	CFU/ml			
	Negative control	Replicate 1	Replicate 2	Replicate 3
Leuconostoc spp.	20 000 000	22 000 000	19 000 000	23 000 000
Lactococcus lactis spp. *lactis* biovar. diacetylactis	85 000 000	82 000 000	79 000 000	90 000 000
Lactococcus lactis spp. *lactis/cremoris*	570 000 000	510 000 000	530 000 000	520 000 000

Table 17. Colony forming units per ml (CFU/ml) in negative control and replicates 1, 2 and 3 of starter culture C inoculated in milk containing 100 ppb oxytetracycline

Bacteria	CFU/ml			
	Negative control	Replicate 1	Replicate 2	Replicate 3
Leuconostoc spp.	23 000 000	29 000 000	28 000 000	28 000 000
Lactococcus lactis spp. *lactis* biovar. diacetylactis	120 000 000	78 000 000	82 000 000	80 000 000
Lactococcus lactis spp. *lactis/cremoris*	460 000 000	360 000 000	350 000 000	380 000 000

Table 18. Colony forming units per ml (CFU/ml) in negative control and replicates 1, 2 and 3 of starter culture C inoculated in milk containing 100 ppb trimethoprim-sulfadiazine

Bacteria	CFU/ml			
	Negative control	Replicate 1	Replicate 2	Replicate 3
Leuconostoc spp.	16 000 000	20 000 000	17 000 000	24 000 000
Lactococcus lactis spp. *lactis* biovar. diacetylactis	79 000 000	60 000 000	70 000 000	65 000 000
Lactococcus lactis spp. *lactis/cremoris*	460 000 000	400 000 000	470 000 000	410 000 000

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